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# NEW DEVELOPMENTS IN ANABIOSIS

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/Although the author emphasizes the economic aspects of the application of deep-freezing techniques in the breeding of animals, the methods he developed may prove to be of equal importance in other fields, such as bacteriology, conservation of tissues for implantation, etc./

About 50 years ago, the prominent Russian scientist P. N. Bakhmet'ev began his famous studies of anabiosis on insects. After chilling butterflies to temperatures of a few degrees below freezing, he established that the insects, numbed by the cold, and apparently lifeless, revived upon warming. Bakhmet'ev was convinced that all liquid in the organism freezes at a temperature of -5°C to -10°C, and thus, as it seemed to him, completely stops the metabolism. He therefore concluded that a definite reviving of dead insects had taken place. As a matter of fact, their return to life was from a special anabiotic condition, by which all life processes are suspended, but in which, as distinguished from death, the capacity for restoration to life is preserved. This condition can best be expressed by the phrase "Neither life nor death."

The idea of anabiosis as a temporary but complete cessation of life allowed Bakhmet'ev to set fantastic tasks before biological science. As a matter of fact, if life were halted, the slow wear and tear, the aging of the organisms which goes on all through life, would also cease. It would mean that it is possible to lengthen the life of animals and even of man for an indefinitely long time; he need only be brought into the anabiotic state and kept there for many years (Bakhmet'ev wrote "the required number of years").





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Bakhmet'ev's experiments aroused lively interest, not only among scientific specialists, but also among wide circles of the general public. An indication of this interest was, among other things, the appearance of a number of science-fiction stories, whose hero had "frozen," i.e., fallen into the anabiotic state for hundreds or thousands of years, to emerge into the world of the future after his awakening.

From the very beginning, Bakhmet'ev imparted practical purposes to his investigation. He was interested, above all, in the possibility of practical application of anabiosis in medicine, agriculture, and the refrigeration industry. In 1913, the year of his death, he planned to conduct experiments with bats on the treatment of tuberculosis by cold. He even dreamed of the use of anabiosis for preserving livestock during winter fodder shortages.

The work of the Soviet scientists I. N. Kalabukhov, I. L. Sakharov, L. K. Lozina-Lozinskiy, and others introduced substantial changes in Bakhmet'ev's theory of anabiosis. It was shown that at such comparatively high temperatures as 5 or even 10°C below zero by far not all the water in the body of animals freezes, and that fairly noticeable metabolism takes place. Consequently, one should not speak of a complete absence of life. On the other hand, I. N. Kalabukhov's investigations showed that bats did not revive after having been actually completely frozen. The experiments of L. K. Lozina-Lozinskiy, who froze corn-moth caterpillars to the extremely low temperature of -80°C, established that anabiosis represents a sharp depression of all life processes. After thawing, the caterpillars revived.

The term anabiosis, implying a new kind of state of the organism -- between life and death -- is not always correctly applied. Since there cannot be any completely static matter, there cannot be any state of suspended life.

The basic idea of Bakhmet'ev's theory, as we shall see later, has proved to be most fruitful: the use of low temperature for increasing the life sp[illegible] of organisms and cells. Low temperature greatly lowers the level of the life processes going on in the organism, thus delaying its aging, and moving back the moment of inevitable death.

However, carrying out this idea in practice has not turned out so easy as all that. The majority of animals (those generally called warm-blooded animals) cannot withstand chilling and frequently perish from it. If the temperature during chilling does not drop lower than the freezing point of the protoplasm, then death occurs as a result of the disarrangement of life activity processes caused by the fact that individual processes, at reduced temperatures, do not slow down at the same rate. On deeper cooling, the water in the protoplasm and in the various body fluids begins to freeze. The removal of water from the proteins of the protoplasm brings about their coagulation and, as a result, the organism perishes. In addition, mechanical damage to the protoplasm caused by crystalline ice in it can also play a great role.

Thus, Bakhmet'ev's dream of the possibility of freezing and reviving large farm animals remains unfeasible for the present. It should be stated that with the victory of the collective structure of agriculture in our country, the question of preserving livestock during the winter has lost its former importance; fodder shortage, the scourge of animal husbandry in Tsarist Russia, has become a thing of the past.

Nevertheless, Bakhmet'ev's theory has found brilliant use in socialist animal husbandry, although in an entirely different form from that he imagined.

Already at the end of the past century, Bakhmet'ev's contemporary, the well-known Russian scientist I. I. Ivanov, established a method for artificially inseminating farm animals. Under the conditions of Tsarist Russia, the method could not develop properly. The rebirth of the method of artificial insemination did not take place until the Soviet era. Worked out in detail and developed by a group of Soviet scientists led by V. K. Milovanov, this method was transformed into a powerful means for improving the quality of socialist cattle breeding, making it possible to obtain from prize rams, bulls, and stallions several times more offspring than by normal mating.

The first investigators in the field of artificial insemination assumed that the sperm should be kept at the same temperature as that of the animal (about 40°C) up to the moment the female is inseminated. However, it was quickly established that the spermatozoa can withstand much lower temperatures, down to 0°C, without ill effects and that it is possible only at precisely those temperatures (in full agreement with Bakhmat'ev's views) to keep them for a long time outside the body. Increased temperature speeds up the metabolism, and thus the spermatozoa use up their resources quickly; they age and deteriorate, and finally die.

The method of keeping livestock sperm at temperatures of 0°C, as developed by Soviet scientists, allows keeping the sperm cells alive outside the body for a period of several weeks. However, after a few days, the sperms, while retaining their ability to move lively, lose their ability to fertilize egg cells. The reason for this has not yet been explained, but obviously at a temperature of 0°C appreciable life processes still take place in the sperm cells, leading to the accumulation of dissimilation products and thus to the gradual self-poisoning of the spermatozoa.

The rate of metabolism processes can be slowed down by greatly reducing the temperatures. But a new difficulty crops up here: at a temperature of -0.6°C, crystalline freezing of the sperm fluids sets in, and the spermatozoa die. It is true that the cooling of sperm fluids to several degrees below zero is sometimes successful, but if the temperature is lowered further, the sperm will freeze.

At the same time, it has been known for a long time that some biological organisms surprisingly resist cold. At first glance, these facts are a complete contradiction of what has been stated above about the freezing of organisms. The seeds of plants, microscopic animals, Rotifera, Tardigrada, and Nematoda, and also many bacteria can withstand immersion in liquid oxygen (-183°C), liquid nitrogen (-196°C), and even in liquid helium (approximately -270°C) without losing their ability to revive when reheated. It is essential to recall that similar experiments interested I. V. Michurin. In 1915, he wrote: "There is one great regrettable defect in all these experiments, namely, the fact that the effect of such low temperatures on plants growing from seeds subjected to these experiments has remained unknown to us. The fact that the life of the seed is preserved does not serve as an indication that it will not suffer the loss of some of its other properties." Until now, this highly important and profound remark has unfortunately not been taken into account by researchers studying the effect of low temperatures on life phenomena.

The spermatozoa of frogs and roosters show an amazing resistance to cold. They are able to withstand the temperatures of liquefied gases (under conditions of preliminary treatment, consisting of partial dehydration of the cells). However, rooster spermatozoa will die if frozen at temperatures much below -6°C.

To explain this paradoxical phenomenon, the hypothesis of the so-called vitrifaction of the protoplasm was advanced. This hypothesis, based on the physics investigations of Professor G. A. Tanman, is outlined below.

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In ordinary freezing, water goes from the liquid state to the solid crystalline (which, as we have seen, is the reason for the damage to the cell protoplasm). But under certain conditions, water (or protoplasm, which always contains a large percentage of water) can freeze without the formation of crystals, forming an amorphous vitreous mass.

The crystallization process takes place at very great speed and within a comparatively narrow temperature range, immediately around the freezing point of the liquid. When the temperature is lowered several tens of degrees below the freezing point, the crystallization rate drops nearly to zero. This circumstance is decisive for the successful vitrifaction of liquids. To avoid crystalline freezing, a small volume of liquid has to be frozen at maximum speed. By this method, it is possible to jump over the dangerous temperature zone in which the processes of crystallization proceed at a high rate of speed, and to reach the zone of vitrifaction safely.

Pure water in the vitreous state can be obtained only with great difficulty. Thus, the more dehydrated an object is, the easier it is to vitrify it.

In the light of the above statements, it becomes understandable why rooster sperms, for example, can withstand the temperature of liquid hydrogen, but will die at $-6^{\circ}C$. Obviously, in the first case, the protoplasm vitrifies as a result of the rapid cooling, and does not form ice crystals. Desiccated Tardigrada or plant seeds can withstand low temperatures because of their low content of water that can undergo crystalline freezing.

In several studies (Graevskiy, Layt, and others), based on the vitrifaction of the protoplasm, various small biological organisms were subjected to deep freezing by immersion in liquid air, liquid nitrogen, and other liquefied gases. The experiments with spermatozoa of frogs and roosters were the most successful. As already stated, these spermatozoa can withstand deep freezing, after preliminary dehydration by strong salt and sugar solutions. Nevertheless, the recovery of motion by the spermatozoa can in no case be construed as an indication that they have kept their fertilizing ability. Attempts by American researchers to obtain offspring from the insemination of females with frozen sperm have been unsuccessful. It is true that, in one case, by the insemination of hens with sperm kept at a temperature of $-78^{\circ}C$ for one hour, some fertilized eggs were obtained, but the embryos died after 10-15 h of development. Thus, this experiment also did not establish that sperms which had withstood cooling to $-78^{\circ}C$ remain physiologically perfect.

As for the spermatozoa of domestic mammals, they did not withstand deep freezing in any experiment and died completely.

In 1947, the author began experiments on the deep freezing of the sperm or rams, bulls, stallions, and rabbits.

After long unsuccessful attempts (at which time it was established that the deep-freezing methods suggested by American authors were unsuitable), a method was finally found by which a portion of rabbit spermatozoa could be made to withstand cooling to temperatures of $-78^{\circ}C$ (the temperature of dry ice), $-183^{\circ}C$ (liquid oxygen), and $-196^{\circ}C$ (liquid nitrogen). To be sure, in this case, from 70 to 90 percent of the spermatozoa died at the moment of cooling, but, on the other hand, the remainder could be kept in the refrigerated store for a long period (32 days), and the percentage of surviving spermatozoa upon rewarming was maintained at one and the same level, regardless of the length of storage. (This refers to liquid oxygen; keeping them in dry ice for prolonged periods results in a slight lowering of the percentage of surviving spermatozoa.)

The exploratory experiments showed the essential practicability of this method in the cooling and thawing of sperm of bulls, stallions, and rams. The results of the experiments confirmed the general correctness of the vitrifaction hypothesis: the spermatozoa withstood deep freezing to temperatures of -78°C and -196°C, but all of them died at much higher temperatures (-15°C and -20°C), demonstrating that crystalline freezing of the protoplasm obviously took place in the second case.

An important and interesting question had to be decided: Do the spermatozoa retain their fertilizing ability subsequent to cooling? To decide this question, an experiment was carried out on rabbits (five males and 36 females of various breeds) To exclude all doubts about the authenticity of the results obtained, various precautions were taken. The fertile female rabbits were kept in individual cages in a special room, while the male rabbits were kept in another building. The researcher himself carried out the insemination of the female rabbits, and he was the only one who knew the breed and the number of the male rabbit with whose refrigerated sperms he had inseminated a certain female rabbit. The use in the experiment of rabbits of different color and length of fur facilitated the control of the experiment and at the same time excluded the possibility of objections that after refrigeration the spermatozoa can lose the ability to transmit the characteristics of the father. Thus, in the insemination of white long-haired Angora female rabbits with the sperm of a short-haired male rabbit the color of a grey hare, short-haired rabbits with exactly the same color of fur as their father's and resembling him in build were obtained.

Several female rabbits were inseminated two or three times in the course of the experiment (i.e., they were used in several successive series of experiments).

The appended table shows that the percentage of fertilization is fairly high (if the unusual conditions of the experiment are taken into consideration) and that it does not become lower as the length of time the sperm is stored is increased. The fourth series of experiments is an exception; the poor quality (dirtiness) of the dry ice used in this series might be the reason for the inferior results. It is not impossible that at such comparatively high temperatures as -78°C, slow recrystallization of the vitrified solidified protoplasm of the spermatozoa, i.e., a change from the vitreous to the crystalline state might have taken place.

The rabbits obtained showed no noticeable deviation from the normal in build, color, length of fur, dynamics of growth, and of the time in which they reached sexual maturity. They were observed for 4-6 months of growth, and in some cases, for a year and a half. Some of the young male rabbits were later mated with young female rabbits. The offspring were completely normal. Up to now, some of the rabbits have produced three to four litters. In one case, a unique second "refrigerated" generation was obtained: one of the young female rabbits, obtained from insemination with refrigerated sperm, was again impregnated with refrigerated sperm, and produced normal offspring.

The obtaining of these offspring is the best proof the full physiological worth of the sperm cells which remained alive after refrigeration.

One might ask what keeps the strong temperature effect exerted on the sex cells from having any noticeable influence on the quality of the offspring. Is this not in contradiction to the teachings of Michurin on the decisive influence of the environment on the inherited properties of the organism?

The best answer to this question is given by T. D. Lysenko in his <u>On the Situation in Biological Science, 1948</u>: "The reason for the change of the nature of living bodies is the variation in the type of assimilation, in the type of metabolism. Know how to change the type of metabolism of the living body, and you will change the heredity."

We used a method of extremely fast, almost instantaneous cooling, which led to such a low level of the metabolism processes that a change in the type of metabolism became unlikely. At the temperature of liquid oxygen, the life processes in the sperm which has been transformed into a piece of very solid ice are slowed down to such a degree that it is difficult to discover them. From that viewpoint, one must expect much greater hereditary changes as a result of storing sperm cells at much higher temperatures, such as +20°C, +10°C, and 0°C, where the conditions of the environment are changed as compared to the usual, and where the life processes take place at a rather high level, so that they might lead to a change in the type of metabolism.

The results of our experiments are a new refutation of the pseudoscientific views of the Morganist geneticists who consider the effect of low temperatures, together with X-rays, one of the methods for bringing about so-called mutations (clear hereditary changes of organisms). As was to be expected, we did not obtain any such mutations at all.

Thus, the data given show for the first time the possibility of fertilization and of obtaining normal offspring by means of spermatozoa which have remained for a long time at temperatures of liquid oxygen and of dry ice. Obviously, our work is only the first step in this field and requires further study.

At present, it is too early to speak about quick application of the new method of storing sperm of farm animals. However, it is necessary to mention some prospects for the future.

Lengthening of the periods of storage of the sperm (these periods may in all likelihood be very long, many months or even years) and lengthening of the period of retention of fertilizing ability of the spermatozoa, which is also likely, open new possibilities for our agriculture. In a recent report, Academician T. D. Lysenko placed before Soviet science the problem of lengthening the time of use of the most valuable breeding stock, which is a golden fund for the improvement of socialist animal husbandry. The method described above might be a brilliant solution to this problem. We even visualize the fantastic possibility of obtaining offspring from rams or bulls long dead, whose sperm cells have retained their fertilizing ability while lying for months in liquid oxygen or nitrogen.

The method given here can be of great importance toward transporting sperms over great distances.

In conclusion, it is necessary to dispel the reader's doubt concerning the expense incurred in storing sperms at extremely low temperatures. Liquid oxygen and dry ice, produced by our industry on a large scale, are available at comparatively low cost, so that the expenses involved in storing sperms would not be excessive.

Results of Insemination

| Series of Expt | Refrigerant | Per of Storage of Sperm | Females Insemi-nated | No of Litters | Percent of Females Having Litters | No of Young Born | Of These: Alive | Of These: Still-born |
|---|---|---|---|---|---|---|---|---|
| 1 | Liquid $O_2$ | 10 min to 42 hr | 33 | 22 | 66.7 | 104 | 96 | 8 |
| 2 | Liquid $O_2$ | 14-15 da | 14 | 9 | 64.3 | 46 | 38 | 8 |
| 3 | Solid $CO_2$ | Approx 21 da | 5 | 5 | 100.0 | 16 | 16 | -- |
| 4 | Solid $CO_2$ | 31-32 da | 9 | 3 | 33.3 | 8 | 7 | 1 |
| | | Total | 61 | 39 | -- | 174 | 157 | 17 |
| | | Average | -- | -- | 66.1 | -- | -- | -- |

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